Brief Description of the Drawings

Figure 1 shows the nucleic acid and amino acid sequence of the "Klenow fragment" of Chy polymerase designated Δ Chy.

Fig. 2 shows the reverse transcriptase activity of Δ Chy in dependence of magnesium and manganese salt.

Figure 3 shows the reverse transcription and amplification of a 997 bp fragment of the ß-Actin gene from total mouse liver RNA using Δ Chy and the Expand HiFi-System and decreasing amounts of RNA.

Figure 4 shows the reverse transcription and amplification of a 997 bp fragment of ß-actin from total mouse liver RNA in comparison to Tth polymerase. Reverse transcription was either coupled with amplification ("one tube") using the Expand HiFi-System from Boehringer Mannheim, or after reverse transcription the Expand HiFi-System from Boehringer Mannheim was added to the reaction mixture for the subsequent amplification reaction ("two tube").

Figure 5 shows the reverse transcription and amplification of a 1.83 kb fragment of Dystrophin from total human muscle RNA.

Figure 6 shows the reverse transcription and amplification of a 324 bp fragment of ß-actin from total mouse liver RNA with various amounts of Chy polymerase and various incubation times.

Figure 7 shows schematically the construction of the clone encoding Δ Chy from the clone encoding the wild type gene.

Detailed Description of the Invention

In referring to a peptide chain as being comprised of a series of amino acids "substantially or effectively" in accordance with a list offering no alternatives within itself, we include within that reference any versions of the peptide chain bearing substitutions made to one or more amino acids in such a way that the overall structure and the overall function of the protein composed of that peptide chain is substantially the same as - or undetectably different to - that of the unsubstituted version. For example it is generally possible to exchange alanine and valine without greatly changing the properties of the protein, especially if the changed site or sites are at positions not critical to the morphology of the folded protein.

3'-5' exonuclease activity is commonly referred as "proofreading" or "editing" activity of DNA polymerases . It is located in the small domain of the large fragment of Type A polymerases. This activity removes mispaired nucleotides from the 3' end of the primer terminus of DNA in the absence of nucleoside triphosphates (Kornberg A. and Baker T. A.(1992) DNA Replication W. H. Freemann & Company, New York). This nuclease action is suppressed by deoxynucleoside triphosphates if they match to the template and can be incorporated into the polymer.
The 3'- 5' exonuclease activity of the claimed DNA polymerase can be measured as degradation or shortening of a 5'-digoxygenin-labeled oligonucleotide annealed to template DNA in the absence or presence of deoxyribonucleoside triphosphates or on DNA fragments in the absence or presence of deoxyribonucleoside triphosphates.

Carboxydothermus hydrogenoformans DNA polymerase is the first DNA polymerase isolated from thermophilic eubacteria with a higher activity in the presence of magnesium ions than in the presence of manganese ions as shown in figure 2. The reverse transcriptase activity in dependence of magnesium is of advantage since the DNA polymerases synthesize DNA with higher fidelity in the presence of magnesium than in the presence of manganese (Beckmann R. A. et al. (1985) *Biochemistry* **24**, 5810-5817; Ricchetti M. and Buc H. (1993) *EMBO J.* **12**, 387-396). Low fidelity DNA synthesis is likely to lead to mutated copies of the original template. In addition,

Mn^{2+} ions have been implicated in an increased rate of RNA degradation, particularly at higher temperatures and this can cause the synthesis of shortened products in the reverse transcription reaction.

The DNA sequence (SEQ ID No.: 10) of Δ Chy polymerase and the derived amino acid sequence (SEQ ID No.: 11) of the enzyme are shown in figure 1. The molecular weight deduced from the sequence is 70.3 kDa, in SDS polyacrylamide gel electrophoresis however Δ Chy polymerase has an electrophoretic mobility of approximately 65 kDa.

The Δ Chy DNA Polymerase has reduced 5'-3' - exonuclease activity and has a temperature optimum at 72°C and exhibits reverse transcriptase activity at temperatures between 50°C and 75°C.

When using Δ Chy DNA Polymerase obtainable from *Carboxydothermus hydrogenoformans* having reduced 5'-3' - exonuclease activity in RT-PCR as reverse transcriptase with subsequent PCR reaction using Taq-polymerase as PCR enzyme a remarkable high sensitivity is achieved (Figure 3). The sensitivity of Δ Chy DNA Polymerase in RT-PCR is higher than the sensitivity of e.g. DNA polymerase from *Thermus thermophilus* (Tth polymerase) (Example 3, Figure 4). Δ Chy DNA Polymerase also exhibits high sensitivity by amplifying a 1.83 kB fragment from total RNA from human muscle (Figure 5). The error rate of Δ Chy DNA Polymerase is 1.58×10^{-4} mutations per nucleotide per cycle and is therewith lower than the error rate of Tth Polymerase which is 2.37×10^{-4} mutations per nucleotide per cycle. This results in higher fidelity of Δ Chy DNA polymerase in comparison to Tth Polymerase.

Carboxydothermus hydrogenoformans was isolated from a hot spring in Kamchatka by V. Svetlichny. A sample of *C. hydrogenoformans* was deposited on the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) under the terms of the Budapest Treaty and received Accession Number DSM 8979. The thermostable polymerase isolated from

Carboxydothermus hydrogenoformans has a molecular weight of 100 to 105 KDa. The thermostable enzyme possesses 5′-3′ polymerase activity, a 3′-5′- exonuclease activity and a reverse transcriptase-activity which is Mg^{++}-dependent. The thermostable enzyme may be native or recombinant and may be used for first- and second-strand cDNA synthesis, in cDNA cloning, DNA sequencing, DNA labeling and DNA amplification.

For recovering the native protein *C.hydrogenoformans* may be grown using any suitable technique, such as the technique described by Svetlichny et al. (1991) *System. Appl. Microbiol.* **14**, 205-208. After cell growth one preferred method for isolation and purification of the enzyme is accomplished using the multi-step process as follows:

The cells are thawed, suspended in buffer A (40 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 0.4 M NaCl, 10 mM Pefabloc) and lysed by twofold passage through a Gaulin homogenizer. The raw extract is cleared by centrifugation, the supernatant dialyzed against buffer B (40 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 10 % Glycerol) and brought onto a column filled with Heparin-Sepharose (Pharmacia). In each case the columns are equilibrated with the starting solvent and after the application of the sample washed with the threefold of its volume with this solvent. Elution of the first column is performed with a linear gradient of 0 to 0.5 M NaCl in Buffer B. The fractions showing polymerase activity are pooled and ammonium sulfate is added to a final concentration of 20 %. This solution is applied to a hydrophobic column containing Butyl-TSK-Toyopearl (TosoHaas). The column is eluted with a falling gradient of 20 to 0 % ammonium sulfate. The pool containing the activity is dialysed and again transferred to a column of DEAE-Sepharose (Pharmacia) and eluted with a linear gradient of 0-0.5 M NaCl in buffer B. The fourth column contains Tris-Acryl-Blue (Biosepra) and is eluted as in the preceding case. Finally the active fractions are dialyzed against buffer C (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7.0 mM 2-mercaptoethanol, 100 mM NaCl, 50 % Glycerol.

DNA polymerase activity was measured by incorporation of digoxigenin-labeled dUTP into the synthesized DNA and detection and quantification of the incorporated digoxigenin essentially

according to the method described in Höltke, H.-J.; Sagner, G; Kessler, C. and Schmitz, G. (1992) *Biotechniques* **12**, 104 -113. The reaction is performed in a reaction volume of 50 µl containing 1 or 2 µl of diluted (0.05 U - 0.01 U) DNA polymerase and 50 mM Tris-HCl, pH 8.5; 12.5 mM $(NH_4)_2SO_4$; 10 mM KCl; 5 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 33 µM dNTPs; 200 µg/ml BSA; 12 µg of DNAse I-activated DNA from calf thymus and 0.036 µM digoxigenin-dUTP.

The samples are incubated for 30 min. at 72°C, the reaction is stopped by addition of 2 µl 0.5 M EDTA, and the tubes placed on ice. After addition of 8 µl 5 M NaCl and 150 µl of Ethanol (precooled to -20°C) the DNA is precipitated by incubation for 15 min. on ice and pelleted by centrifugation for 10 min at 13000 x rpm and 4°C. The pellet is washed with 100 µl of 70% Ethanol (precooled to -20°C) and 0.2 M NaCl, centrifuged again and dried under vacuum.

The pellets are dissolved in 50 µl Tris-EDTA (10 mM/0.1 mM; pH 7.5). 5 µl of the sample are spotted into a well of a nylon membrane bottomed white microwave plate (Pall Filtrationstechnik GmbH, Dreieich, FRG, product no: SM045BWP). The DNA is fixed to the membrane by baking for 10 min. at 70°C. The DNA loaded wells are filled with 100 µl of 0.45 µm-filtrated 1 % blocking solution (100 mM maleic acid, 150 mM NaCl, 1 % (w/v) casein, pH 7.5). All following incubation steps are done at room temperature. After incubation for 2 min. the solution is sucked through the membrane with a suitable vacuum manifold at -0.4 bar. After repeating the washing step, the wells are filled with 100 µl of a 1:10,000-dilution of Anti-digoxigenin-AP, Fab fragments (Boehringer Mannheim, FRG, no: 1093274) diluted in the above blocking solution. After incubation for 2 min. and sucking this step is repeated once. The wells are washed twice under vacuum with 200 µl each time washing-buffer 1 (100 mM maleic-acid, 150 mM NaCl, 0.3 %(v/v) Tween^{TM} 20, pH 7.5). After washing another two times under vacuum with 200 µl each time washing-buffer 2 (10 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) the wells are incubated for 5 min. with 50 µl of CSPD^{TM} (Boehringer Mannheim, no: 1655884), diluted 1:100 in washing-buffer 2, which serves as a chemiluminescent substrate for the alkaline phosphatase. The solution is sucked through the membrane and after 10 min. incubation the

RLU/s (Relative Light Unit per second) are detected in a Luminometer e.g. MicroLumat LB 96 P (EG&G Berthold, Wilbad, FRG).

With a serial dilution of *Taq* DNA polymerase a reference curve is prepared from which the linear range serves as a standard for the activity determination of the DNA polymerase to be analyzed.

The Determination of reverse transcriptase activity is performed essentially as described for determination of DNA polymerase activity except that the reaction mixture consists of the following components: 1 µg of polydA-$(dT)_{15}$, 33 µM of dTTP, 0.36 µM of digoxigenin-dUTP, 200 mg/ml BSA, 10 mM Tris-HCl, pH 8.5, 20 mM KCl, 5 mM $MgCl_2$, 10 mM DTE and various amounts of DNA polymerase. The incubation temperature used is 50°C.

Isolation of recombinant DNA polymerase from *Carboxydothermus hydrogenoformans* may be performed with the same protocol or with other commonly used procedures.

The production of a recombinant form of *Carboxydothermus hydrogenoformans* DNA polymerase generally includes the following steps: chromosomal DNA from *Carboxydothermus hydrogenoformans* is isolated by treating the cells with detergent e.g. SDS and a proteinase e.g. Proteinase K. The solution is extracted with phenol and chloroform and the DNA purified by precipitation with ethanol. The DNA is dissolved in Tris/EDTA buffer and the gene encoding the DNA polymerase is specifically amplified by the PCR technique using two mixed oligonucleotides (primer 1 and 2). These oligonucleotides, described by SEQ ID No.: 1 and SEQ ID No.: 2, were designed on the basis of conserved regions of family A DNA polymerases as published by Braithwaite D. K. and Ito J. (1993) *Nucl. Acids Res.* 21, 787 - 802. The specifically amplified fragment is ligated into an vector, preferably the pCR™II vector (Invitrogen) and the sequence is determined by cycle-sequencing. Complete isolation of the coding region and the flanking sequences of the DNA polymerase gene can be performed by restriction fragmentation of the *Carboxydothermus hydrogenoformans* DNA with another restriction enzyme as in the first round of screening and by inverse PCR (Innis et al., (1990) PCR Protocols; Academic Press, Inc.,

219-227). This can be accomplished with synthesized oligonucleotide primers binding at the outer DNA sequences of the gene part but in opposite orientation. These oligonucleotides described by SEQ ID Nos. 3 and 4 , were designed on the basis of the sequences which were determined by sequencing of the first PCR product described above. As template DNA from *Carboxydothermus hydrogenoformans* is used which is cleaved by restriction digestion and circularized by contacting with T4 DNA ligase. To isolate the coding region of the entire polymerase gene, another PCR is performed using primers as shown in SEQ ID Nos. 5 and 6. The complete DNA polymerase gene is amplified directly from genomic DNA with primers suitable for introducing ends compatible with the linearized expression vector.

SEQ ID No. 1:

Primer 1: 5′-CCN AAY YTN CAR AAY ATH-3′

SEQ ID No. 2:

Primer 2: 5′-YTC RTC RTG NAC YTG-3′

SEQ ID No. 3:

Primer 3: 5'-GGG CGA AGA CGC TAT ATT CCT GAG C-3'

SEQ ID NO. 4:

Primer 4: 5'-GAA GCC TTA ATT CAA TCT GGG AAT AAT C-3'

SEQ ID NO. 5:

Primer 5: 5'-CGA ATT CAA TCC ATG GGA AAA GTA GTC CTG GTG GAT-3'

SEQ ID NO. 6:

Primer 6: 5'-CGA ATT CAA GGA TCC TTA CTT CGC TTC ATA CCA GTT-3'

The gene is operably linked to appropriate control sequences for expression in either prokaryotic or eucaryotic host/vector systems. The vector preferably encodes all functions required for transformation and maintenance in a suitable host, and may encode selectable markers and/or control sequences for polymerase expression. Active recombinant thermostable polymerase can be produced by transformed host cultures either continuously or after induction of expression. Active thermostable polymerase can be recovered either from host cells or from the culture media if the protein is secreted through the cell membrane.

The use of a plasmid as an appropriate vector has shown to be advantageous, particularly pDS56 (Stüber, D., Matile, H. and Garotta, G. (1990) Immunological Methods, Letkovcs, I. and Pernis, B., eds). The plasmid carrying the Carboxydothermus hydrogenoformans DNA polymerase gene is then designated pAR4.

According to the present invention the use of the E. coli strain BL21 (DE3) pUBS520 (Brinkmann et al., (1989) Gene 85, 109-114) has shown to be advantageous. The E.coli strain BL 21 (DEB) pUBS 520 transformed with the plasmid pAR4 is then designated AR96 (DSM No 11179).

The mutant ΔChy was obtained by deletion of an N-terminal fragment of the recombinant wild type *Carboxydothermus hydrogenoformans* DNA polymerase using inverse PCR (Innis et al., (1990) PCR Protocols; Academic Press, Inc., p 219-227). The reverse primer used is complementary to the cloning site of the expression vector pDS56 (Stüber, D., Matile, H. and Garotta, G. (1990) Immunological Methods, Letkovcs, I. and Pernis, B., eds.) at the Nco I restriction site (bases 120-151) and has the sequence:

SEQ ID No. 7:

Primer 7: 5'-CGG TAA ACC CAT GGT TAA TTT CTC CTC TTT AAT GAA TTC-3'.

This primer contains additional 7 bases at the 5' end to ensure a better binding of the Nco I restriction enzyme in the subsequent restriction enzyme cleavage. The second (forward) primer was complementary to bases 676-702 of the wild type gene and has the sequence:

SEQ ID No. 8:

Primer 8: 5'-CGG GAA TCC ATG GAA AAG CTT GCC GAA CAC GAA AAT TTA-3'

The forward primer also contained an additional Nco I restriction site and additional 7 bases at the 5'-end. Plasmid pDS56 DNA containing the polymerase-gene of *Carboxydothermus hydrogenoformans* at the Nco I/BamHI restriction sites was used as template for PCR. The PCR reaction was performed on the circular plasmid DNA pAR4. The fragment encoding the mutated *Carboxydothermus hydrogenoformans* DNA polymerase (Δ Chy) and the vector DNA were amplified as linear DNA by PCR using the Expand High Fidelity PCR System (Boehringer Mannheim) according to the supplier's specifications (Fig. 7). The length of the gene encoding Δ Chy is 1821 bp.

Amplification (Perkin Elmer GeneAmp 9600 thermocycler) was carried out with the following conditions: 2 min 94°C, (10 sec 94°C; 30 sec 65°C; 4 min 68°C) x 10; (10 sec 94°C; 30 sec 65°C; 4 min 68°C) + cycle elongation of 20 sec for each cycle) x 20; 7 min 72°C;
After PCR the amplified DNA was purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim) and digested with NcoI (3U / µg DNA) for 16 h (Boehringer Mannheim) according to the supplier's specifications.

For extraction with Phenol/Chloroform/Isoamylalcohol (24:24:1) the volume of the sample was raised to 100 µl with TE. After extraction the DNA was precipitated by adding 1/10 volumes of 3M Sodium Acetate, pH 5.2 and 2 volumes of EtOH. The DNA was circularized using the Rapid DNA Ligation Kit (Boehringer Mannheim) according to the supplier's specification. The ligated products were introduced into *E. coli* XL1-Blue by transformation according to the procedure of Chung, C. T. et al. (1989) *Proc. Natl. Acad. Sci.* USA **86**, 2172-2175. Transformants were plated on L-agar containing 100 µg/ml ampicillin to allow selection of recombinants. Colonies were picked and grown in L-broth containing 100 µg/ml ampicillin. Plasmid DNA was prepared with the High Pure Plasmid Isolation Kit (Boehringer Mannheim) according to the supplier's specification. The plasmids were screened for insertions by digestion with NcoI/BamHI. Strains containing the genes of interest were grown in L-broth supplemented with 100 µg/ml ampicillin and tested for the expression of DNA polymerase / reverse transcriptase activity by induction of exponentially growing culture with 1 mM IPTG and assaying the heat-treated extracts (72°C) for DNA polymerase / reverse transcriptase activity as described above (determination of DNA polymerase activity and determination of reverse transcriptase activity).

The present invention provides improved methods for efficiently transcribing RNA and amplifying RNA or DNA. These improvements are achieved by the discovery and application of previously unknown properties of thermoactive DNA polymerases with reverse transcriptase activity.

The enzyme of this invention may be used for any purpose in which such enzyme activity is necessary or desired. In a particularly preferred embodiment, the enzyme catalyzes reverse transcription of RNA which is amplified as DNA by a second DNA polymerase present in the amplification reaction known as RT-PCR (Powell et al. (1987) *Cell* **50**, 831-840). Any ribonucleic acid sequence, in purified or nonpurified form, can be utilized as the starting nucleic acid(s), provided it contains or is suspected to contain the specific nucleic acid sequence desired. The nucleic acid to be amplified can be obtained from any source, for example, from plasmids such as pBR322, from cloned RNA, from natural RNA from any source, including bacteria, yeast,

viruses, organelles, and higher organisms such as plants and animals, or from preparations of nucleic acids made *in vitro*.

RNA may be extracted from blood, tissue material such as chorionic villi, or amniotic cells by a variety of techniques. See, e.g., Maniatis et al., (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) pp. 280-281. Thus the process may employ, for example, RNA, including messenger RNA, which RNA may be single-stranded or double-stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized.

The amplification of target sequences from RNA may be performed to proof the presence of a particular sequence in the sample of nucleic acid to be analyzed or to clone a specific gene. Δ Chy DNA polymerase is very useful for these processes. Due to its 3´-5´exonuclease activity it is able to synthesize products with higher accuracy as the reverse transcriptases of the state of the art.

Δ Chy DNA polymerase may also be used to simplify and improve methods for detection of RNA target molecules in a sample. In these methods Δ Chy DNA polymerase from *Carboxydothermus hydrogenoformans* may catalyze: (a) reverse transcription and (b) second strand cDNA synthesis. The use of DNA polymerase from *Carboxydothermus hydrogenoformans* may be used to perform RNA reverse transcription and amplification of the resulting complementary DNA with enhanced specificity and with fewer steps than previous RNA cloning and diagnostic methods.

Another aspect of the invention comprises a kit for performing RT-PCR comprising Δ Chy polymerase, reaction buffers, nucleotide mixtures, and optionally a thermostable DNA polymerase for detection and amplification of RNA either in a one step reaction or for reverse transcription of the template RNA and subsequent amplification of the cDNA product.

The following examples describe the invention in greater detail:

Example 1

Reverse transcription of a 324 bp ß-actin fragment with Chy wild type DNA Polymerase used as Reverse Transcriptase followed by PCR with Taq-polymerase (Figure 6).

The reaction mixture (20 µl) contained 200 ng total mouse liver RNA, 200 µM dNTP, 10 mM Tris-HCl, pH 8.8, 5 mM DTT, 10 mM 2-mercaptoethanol, 15 mM KCl, 4.5 mM $MgCl_2$, 0.02 mg/ml BSA, 20 pmol of reverse primer (ß-actin reverse: 5'-AAT TCG GAT GGC TAC GTA CAT GGC TG-3' [SEQ ID NO: 9]) and Chy-polymerase 33 units (lanes 1, 4, 7, 10, 13, 16), 13.2 units (lanes 2, 5, 8, 11, 14, 17) and 6.6 units (lanes 3, 6, 9, 12, 15, 18). Reactions were incubated for 5 min (lanes 1 to 6), 10 min (lanes 7 to 12) and 15 min (lanes 13 to 18) at 70°C.
20 µl of the reverse transcription reaction was used as template for PCR (100 µl reaction volume) with Taq-polymerase (Boehringer Mannheim) according to the supplier's specification using 20 pmol of forward and reverse primer (Primer sequence "ß-actin forward": 5'AGC TTG CTG TAT TCC CCT CCA TCG TG-3' [SEQ ID NO: 12], primer sequence "ß-actin reverse": 5'-AAT TCG GAT GGC TAC GTA CAT GGC TG-3' [SEQ ID NO: 9]) and 200 µM dNTP's. Amplification was carried out using the following temperature profile: 2 min 94°C; (10 sec 94°C; 30 sec 60°C; 30 sec 72°C) x 30; 7 min 72°C.

Example 2

Construction of the vector expressing Δ Chy.

The mutant was obtained by deletion of an N-terminal fragment of recombinant wild type *Carboxydothermus hydrogenoformans* DNA polymerase using inverse PCR (Innis et al., (1990) PCR Protocols; Academic Press, Inc., p 219-227). The reverse primer used is complementary to the cloning site of the expression vector pDS56 (Stüber, D., Matile, H. and Garotta, G. (1990) Immunological Methods, Letkovcs, I. and Pernis, B., eds.) at the Nco I restriction site (bases 120-

151) and has the sequence: 5'-CGG TAA ACC CAT GGT TAA TTT CTC CTC TTT AAT GAA TTC-3' (SEQ ID NO: 7). This primer contains additional 7 bases at the 5' end to ensure a better binding of the Nco I restriction enzyme in the subsequent restriction enzyme cleavage. The second (forward) primer, was complementary to bases 676-702 of the wild type gene (sequence: 5'-CGG GAA TCC ATG GAA AAG CTT GCC GAA CAC GAA AAT TTA-3' [SEQ ID NO: 8]). The forward primer also contained an additional Nco I restriction site and additional 7 bases at the 5'-end. Plasmid pDS56 DNA containing the polymerase-gene of *Carboxydothermus hydrogenoformans* at the Nco I/BamHI restriction sites was used as template for PCR. The PCR reaction was performed on circular plasmid DNA pAR4. The fragment of *Carboxydothermus hydrogenoformans* DNA polymerase (ΔChy) and the vector DNA were amplified as linear DNA by PCR using the Expand High Fidelity PCR System (Boehringer Mannheim) according to the supplier's specifications. The length of the gene encoding Δ Chy is 1821 bp.
Amplification (Perkin Elmer GeneAmp 9600 thermocycler) was carried out with the following conditions: 2 min 94°C, (10 sec 94°C; 30 sec 65°C; 4 min 68°C) x 10; (10 sec 94°C; 30 sec 65°C; 4 min 68°C) + cycle elongation of 20 sec for each cycle) x 20; 7 min 72°C.

After PCR the amplified DNA was purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim) and digested with NcoI (3U / µg DNA) for 16 h (Boehringer Mannheim) according to the supplier's specifications. For extraction with Phenol/Chloroform/Isoamylalcohol (24:24:1) the volume of the sample was raised to 100 µl with TE. After extraction the DNA was precipitated by adding 1/10 volumes of 3M Sodium Acetate, pH 5.2 and 2 volumes of EtOH. The DNA was circularized using the Rapid DNA Ligation Kit (Boehringer Mannheim) according to the supplier's specification. The ligated products were introduced into *E. coli* XL1-Blue by transformation according to the procedure of Chung, C. T. et al. (1989) *Proc. Natl. Acad. Sci.* USA 86, 2172-2175. Transformants were plated on L-agar containing 100 µg/ml ampicillin to allow selection of recombinants. Colonies were picked and grown in L-broth containing 100 µg/ml ampicillin. Plasmid DNA was prepared with the High Pure Plasmid Isolation Kit (Boehringer Mannheim) according to the supplier's specification. The plasmids were screened for insertions by digestion with NcoI/BamHI. Strains containing the genes of interest were grown in

L-broth supplemented with 100 µg/ml ampicillin and tested for the expression of DNA polymerase / reverse transcriptase activity by induction of exponentially growing culture with 1 mM IPTG and assaying the heat-treated extracts (72°C) for DNA polymerase / reverse transcriptase activity as described above (determination of DNA polymerase activity and determination of Reverse Transcriptase activity). (Figure 7)

Example 3

Reverse transcription and amplification of a 997 bp fragment of ß-actin from total mouse liver RNA. Comparison of Δ Chy with Tth polymerase in the reverse transcription reaction (Figure 4) either in a coupled RT-PCR reaction ("one tube") or in consecutive steps, reverse transcription, addition of polymerase and amplification of the cDNA product of the first step.

"one tube" system:

The reactions (50 µl) contained 10 mM Tris-HCl, pH 8.8 at 25°C, 15 mM KCl, 2.5 mM $MgCl_2$, 400 µM of each dNTP, decreasing amounts of mouse total RNA (Clonetech) as indicated in the figure, 300 nM of each primer, 60 units of Δ Chy and 3.5 units of the Expand HiFi enzyme mix (Boehringer Mannheim GmbH). All reactions were incubated at 60°C for 30 min (RT step). Amplification followed immediately with following cycle profile (Perkin Elmer GeneAmp 9600 thermocycler): 30 sec. at 94°C; (30 sec at 94°C, 30 sec at 60°C, 1 min. at 68°C) x 10; (30 sec. at 94°C, 30 sec. at 60°C, 1 min. at 68°C + cyle elongation of 5 sec. for each cycle) x 20; 7 min at 68°C;

"two tube" system:

Reverse transcription is performed in 10 mM Tris-HCl, pH 8.8, 15 mM $(NH_4)_2SO_4$, 0.1 % Tween, 4.5 mM $MgCl_2$, 2 % DMSO, 800 µM dNTPs, 300 nmoles of each primer, 60 units of Δ Chy, various amounts of total mouse muscle RNA as indicated in the figure. The reaction was performed in a volume of 25 µl for 30 min at 60°C. 5 µl of this reaction are used for the

amplification with the Expand HiFi-system from Boehringer Mannheim. Amplification was performed with 2.6 units of polymerase mixture in a reaction volume of 25 µl. The following temperature cycling conditions were used: 30 sec. at 94°C, (30 sec. at 94°C, 30 sec at 60°C, 1 min at 68°C) x 10, (30 sec. at 94°C, 30 sec. at 60°C, 1 min at 68°C + cycle elongation for 5 sec for each cycle) X 20.

As a control reaction the same template-primer system was used for RT-PCR with Tth polymerase (Boehringer Mannheim). The reaction was set up according to the supplier's specifications for the "one step" variant.

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